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Time-resolved fluorescence studies on the dihydrolipoyl transacetylase (E_2) component of the pyruvate dehydrogenase complex from *Azotobacter vinelandii*

Roeland Hanemaaijer, Remco Masurel, Antonie J.W.G. Visser, Arie de Kok and Cees Veeger

Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

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The dihydrolipoyl transacetylase (E_2) component of *A. vinelandii* PDC and its lipoyl domain shows similar dynamic properties as revealed with fluorescence anisotropy decay of lipoyl-bound IAANS. The lipoyl domain (32.6 kDa), containing three almost identical subdomains shows a mode of rotation characteristic for a protein of about 30 kDa. A similar rotation is found in E_2, indicating an independent rotational mobility of the whole domain in the multimeric E_2 core (1.6 MDa). No independent rotation of a single lipoyl subdomain (10 kDa) is observed. The E_1 component, in contrast to the E_3 component, shows interaction with the lipoyl domain.

Dihydrolipoyl transacetylase; Pyruvate dehydrogenase complex; Time-resolved fluorescence; Mobility; *(Azotobacter vinelandii)*

1. INTRODUCTION

The pyruvate dehydrogenase complex catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA. The structural core of the *A. vinelandii* complex is composed of four dihydrolipoyl transacetylase (E_2) chains, to which three dimers of pyruvate dehydrogenase (E_1) and one dimer of lipoamide dehydrogenase (E_3) are bound [1]. After removal of these peripheral components the E_2 core aggregates to a cubic 24-meric structure [2,3]. The substrates are transferred between the different components by lipoyllysine residues, which act as swinging arms between the different active sites [4]. In *E. coli* and in *A. vinelandii* E_2 the N-terminal part of the E_2 chain consists of three homologous repeating sequences [5,6]. Each repeat of about 80 amino acid residues contains a lysyl residue that is a potential site for lipoylation. Each repeat is separated from its neighbour by a region of about 20 residues, very rich in alanyl and prolyl residues. After limited proteolysis of E_2 with trypsin this N-terminal part, called lipoyl domain (32.6 kDa), is separated from a part called the catalytic domain, which forms the multimeric core [7]. In *E. coli* it is shown that after limited proteolysis with *Staphylococcus aureus* V8 proteinase the three repeats can be isolated separately as folded subdomains [8]. In other organisms such as Gram-positive bacteria and eukaryotes, and also in the closely related 2-oxoglutarate dehydrogenase complex, only one lipoyl subdomain is found [9–11]. Spin label experiments with the PDC from *E. coli* have demonstrated that the dithiolane ring of the lipoyl group can rotate freely in the complex as is indicated by a rotational correlation time of 0.2–1.0 ns [12]. Also a correlation time of more than 50 ns was found, which was attributed to the rotation of the whole complex (expected correlation time 2 μs). When using the triplet probe eosin-maleimide only mobility of the whole complex was found, showing that the label is folded back to the protein surface [13]. From a lack in energy transfer between the lipoyl domain and the FAD group of E_3 it is suggested that the lipoyllysine residue is too short to serve all catalytic centres [14] and

Correspondence address: A. de Kok, Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

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therefore at least a part of the protein chain around the lipoyl residue is thought to be flexible. Direct evidence for conformational flexibility in E_2 chains was obtained from ^{1}H-NMR spectroscopy [15]. In these experiments the regions, which are very rich in alanyl and prolyl residues, are shown to possess conformational flexibility. Thus, from these experiments it has been suggested that all three subdomains in the lipoyl domain can rotate independently. In this paper it is shown with fluorescence anisotropy decay experiments that, although the alanine-proline rich region may possess internal flexibility, the lipoyl domain moves as a single entity within the large multimeric E_2 core. This movement is restricted by the binding of the peripheral components.

2. MATERIALS AND METHODS

2-(4-Iodoacetamidoanilino)-naphthalene-6-sulfonic acid (IAANS) was obtained from Molecular Probes. Dihydrolipoyl transacetylase (E_2) was isolated from the pyruvate dehydrogenase complex by covalent chromatography on thiol-Sepharose 4B as described previously [16], with modifications according to [7]. The lipoyl domain was obtained after limited proteolysis with trypsin of the E_2 component, which was covalently bound on thiol-Sepharose 4B and purified as described before [7]. Labelling was carried out as follows. E_2 and the lipoyl domain were incubated for 30 min at 4°C in 20 mM Tricine, pH 8.5, containing 20 mM dithiothreitol, and anaerobically dialysed against standard buffer (50 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride). From a 15.9 mM IAANS stock solution in H_2O/ethanol (1 : 1, v/v) a fifteen times molar excess to sulfhydryl groups of the lipoyl moieties was added, and after 30 min the sample was dialysed against standard buffer. From optical density measurements it was observed that over 80% of the lipoyl groups were (doubly) labelled. Since it has been shown [2] that only 2–2.5 of the three potential sites per E_2 chain contain a lipoyl group, it is clear that all present lipoyl groups were (doubly) labelled. No bound label was detected without prior reduction. After labelling the complex activity had totally disappeared, but the E_2 (transacetylase) activity was fully retained. Fluorescence spectra were recorded on an Aminco SPF-500 fluorimeter. Time-resolved fluorescence decay was measured with a system consisting of a frequency-doubled synchronously pumped dye laser for excitation and time-correlated single photon counting in detection. The excitation wavelength was at 310 nm and the emission was monitored via a 450 nm band-pass filter (Balzers K45). Details of the experimental set-up have been described in [17]. All experiments were carried out at 20°C. Data analysis was performed as described in [18]. The order parameter S_1 is defined as $[(\beta_2+\beta_3)/(\beta_1+\beta_2+\beta_3)]^{1/2}$ [19]. The parameter S_2, representing the degree of order only connected with the very slow rotation is defined as $[(\beta_3/\beta_2+\beta_3)]^{1/2}$. The related cone angle θ_c for the order parameter S_1 is obtained from the relation $(S_1)^2 = \frac{1}{2}\cos\theta_c(1+\cos\theta_c)$.

3. RESULTS AND DISCUSSION

The initial fluorescence anisotropy decay curves are shown in figs 1 and 2. Fig.1 shows the experimental and the fitted curve of the fluorescence anisotropy decay of E_2, in fig.2 only the fitted curves of the lipoyl domain, E_2, and E_2 with bound E_1 and E_3 components are shown. For the lipoyl domain the anisotropy decays as a double exponential function with a short (0.6 ns) and a longer (11.4 ns) characteristic time constant. The short component can be ascribed to the motion of the lipoyl group rotating freely around the linkage with the protein chain. This time constant correlates well with the values found previously with spin labels [12]. The longer component can be ascribed to the rotation of the whole domain. The correlation time can be calculated on the basis of an empirical formula relating the correlation time

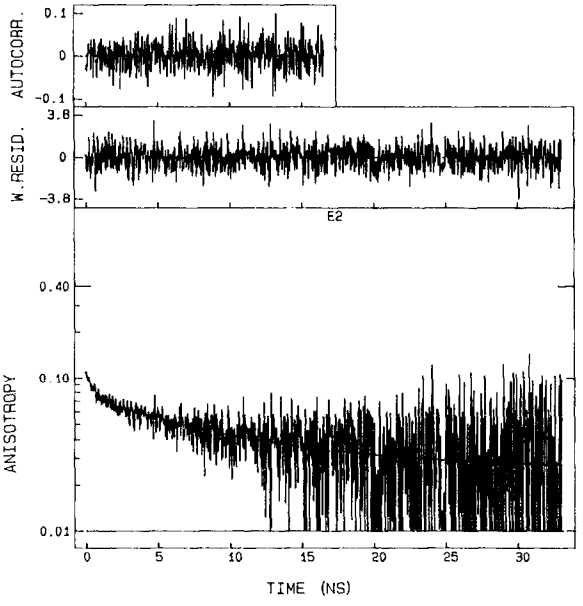

Fig.1. Fluorescence anisotropy decay of E_2. Shown are two curves: the experimental (noisy curve) and the calculated (smooth curve) fluorescence anisotropy decay. The parameters of the triple exponential decay are listed in table 1. The quality of the fit is indicated by the weighted residuals and the autocorrelation function, shown in the upper panels. The statistical parameters are χ^2 = 1.06, and the Durbin-Watson parameter = 2.03.

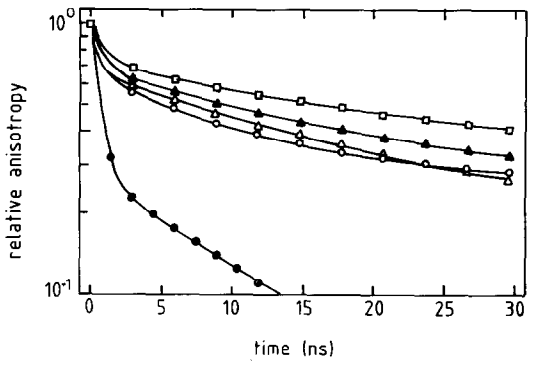

Fig.2. Fluorescence anisotropy decay of the lipoyl domain, E_2, and E_2 complexed with E_1 or E_3. Shown are the fitted curves of (1) the lipoyl domain (●), (2) E_2 (○), (3) $E_2 + E_3$ (▲) in a 4:2 molar concentration, when the E_3 binding site is saturated by E_3, (4) $E_2 + E_3$ (△) in a 4:8 molar concentration, when both the E_3- and E_1 binding sites are saturated by E_3, and (5) $E_2 + E_1$ (□) in a 4:6 molar concentration, when the E_1 binding sites are saturated by E_1. The parameters of the double (1) and the triple (2–5) exponential decays and the standard errors derived from the fits are listed in table 1.

ϕ with M_r of a hydrated, spherical polypeptide at 20°C: $\phi(\text{ns}) = 3.84 \times 10^{-4} \cdot M_r$ [20]. From this formula a correlation time of about 12.5 ns is calculated for the lipoyl domain having an M_r value of 32600 [7]. Despite the fact that the lipoyl domain is thought to have a swollen or extended structure, $f/f_0 = 1.7$ [7], the agreement is rather good. For E_2 the anisotropy decay curve can be described as a triple exponential function with a short (0.4 ns), a longer (10.7 ns) and a very long (600 ns) time constant. The latter is fixed in the fitting procedure and represents the rotation of the whole protein (1.6 MDa). The short component is in the same order as found in the lipoyl domain and accounts for the free rotation of the lipoyl group. The time constant of 10.7 ns will represent the rotation of the lipoyl domain, which is thought to rotate independently of the large E_2 core. From the order parameter S_1 and the related cone angle θ_c that can be derived from a wobbling-in-cone model [19] it is shown that in E_2, where the lipoyl domain is bound to the core, the order increases and the motion of the label is more restricted. This indicates that the motion of the lipoyl domain is not totally independent of the core, but a certain degree of interaction exists. Upon binding of E_1 or E_3 components to the E_2 core no significant difference in correlation times is found (table 1, expts 3–5). From β_3, S_1 and S_2 it is clear that, despite the dissociation of E_2 which takes place upon addition of E_1 (or excess E_3), a significant difference is found in order and in motional restriction upon binding of the E_1 component (expt 5). Upon addition of excess E_3 (expt 4), which is thought to bind on or near the E_1 binding site [1] no significant difference is observed. Obviously some interaction exists between E_1 and the lipoyl domain which is not present between E_3 and the lipoyl domain. This

Table 1

Fluorescence decay parameters of the lipoyl domain, E_2 and E_2 complexed with E_3 or E_1

Expt	Protein[a]	M_r	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)	β_3	ϕ_3[b] (ns)	S_1[c]	θ_c[d]	S_2[e]
1	Lipoyl domain	33000	0.160 ± 0.011	0.62 ± 0.19	0.039 ± 0.002	11.4 ± 1.5	–	–	0.44 ± 0.03	56 ± 2	–
2	E_2	1560000	0.069 ± 0.002	0.38 ± 0.07	0.048 ± 0.002	10.7 ± 1.4	0.024 ± 0.003	600	0.74 ± 0.04	36 ± 3	0.57 ± 0.03
3	E_2+E_3 (4:2)	2160000	0.042 ± 0.004	0.91 ± 0.13	0.051 ± 0.006	18.1 ± 3.7	0.025 ± 0.006	830	0.80 ± 0.11	31 ± 8	0.57 ± 0.11
4	E_2+E_3 (4:8)	660000	0.055 ± 0.008	0.56 ± 0.08	0.060 ± 0.005	16.9 ± 2.5	0.026 ± 0.005	253	0.78 ± 0.10	32 ± 8	0.55 ± 0.08
5	E_2+E_3 (4:6)	860000	0.041 ± 0.004	1.00 ± 0.14	0.047 ± 0.006	20.0 ± 4.7	0.055 ± 0.007	330	0.84 ± 0.10	27 ± 9	0.73 ± 0.09

[a] See legend of fig.2 for details

[b] Fixed in the analysis

[c] From $(S_1)^2 = \dfrac{\beta_2+\beta_3}{\beta_1+\beta_2+\beta_3}$

[d] From $(S_1)^2 = \frac{1}{2}\cos\theta_c(1+\cos\theta_c)$

[e] From $(S_2)^2 = \dfrac{\beta_3}{\beta_2+\beta_3}$

could be related to the observation of Packman et al. [8] who showed that E_3 is able to use lipoamide as a substrate, whereas E_1 needs the intact lipoyl subdomain as substrate. Mobility of lipoyl subdomains has been suggested from ^{1}H-NMR experiments, based on observed conformational mobility of the alanine and proline rich regions. In these fluorescence anisotropy experiments we present a direct indication that the lipoyl domain as a whole contains mobility more or less independent of the large E_2 core. The hinge of this mobility should be located between the lipoyl domain and the catalytic domain (residue 331–381 [6]). In this region also the binding sites for the E_1 and the E_3 components are located [7]. After binding of these components to the E_2 core no dramatic limitation of mobility is observed, suggesting that the hinge of the mobility of the lipoyl domain is located N-terminal of the E_1 and E_3 binding sites. Previous fluorescence anisotropy experiments of the FAD in free and in bound E_3 have shown that E_3, bound to the E_2 core, still possesses a high mobility [21]. This indicates a second hinge C-terminal to the E_3 binding site. This region may correspond with a highly mobile region observed in ^{1}H-NMR experiments near the N-terminus of the catalytic domain [22].

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